(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 December 2006 (07.12.2006)

PCT

(10) International Publication Number
WO 2006/128492 A1

(51) International Patent Classification:
C07K 7/08 (2006.01) *G01N 33/68* (2006.01)

(21) International Application Number:
PCT/EP2005/012530

(22) International Filing Date:
18 November 2005 (18.11.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
05090161.0 2 June 2005 (02.06.2005) EP

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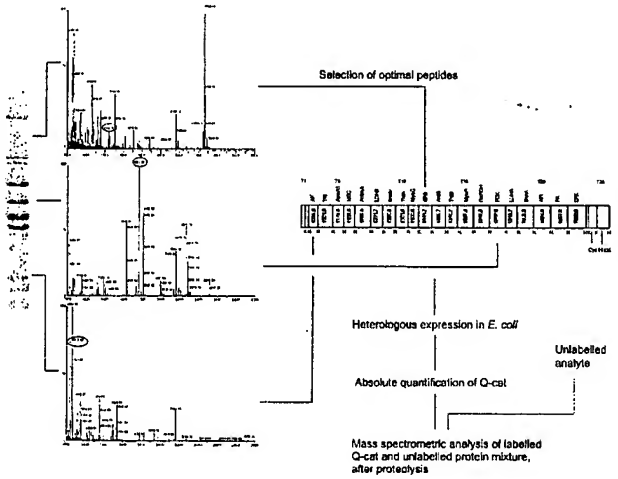
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ARTIFICIAL PROTEIN, METHOD FOR ABSOLUTE QUANTIFICATION OF PROTEINS AND USES THEREOF



(57) Abstract: The invention provides an artificial protein for quantitative analysis of the proteome of a sample, cell or organism, comprising at least two consecutive peptides linked by a cleavage sequence for separating the peptides; a singular marker on one or more peptide for determination of the absolute amount of this fragment; and Nterminal and C-terminal extensions for protection of the peptides; wherein each peptide represents one single protein of the sample, cell or organism and each peptide is in a defined stoichiometry. The invention further provides a collection of peptides, a vector and a kit comprising the artificial protein and a method for quantitative analysis of the proteome.

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Artificial protein, method for absolute quantification of proteins and uses thereof

Field of the invention

The present invention relates to proteomics and more specifically to absolute quantification of proteins.

Background of the invention

The need for absolute quantification in proteomics is becoming increasingly urgent. The most promising method is based on stable isotope dilution involving simultaneous determination of representative proteolytic peptides and stable isotope labeled analogs. The principal limitation to widespread implementation of this approach is the availability of standard signature peptides in accurately known amounts.

The two primary themes in proteomics are protein identification and the comparison of protein expression levels in two physiological or pathological states (comparative proteomics). The long term goal of being able to define the entire proteome of a cell is still unrealized, but the characterization of many thousands of proteins in a single analysis is now attainable.

For proteomics to become a platform technology serving the emergent field of systems biology, there is a pressing need for enhancement of quantification (Righetti, Eur J Mass Spectrom 10 (2004), 335-348). Most comparative proteomics studies deliver *relative* quantification, expressing the changes in amount of a protein in the context of a second cellular state (for example Dunkley, Mol Cell Proteomics (2004); Hoang, J Biomol Tech 14 (2003), 216-233; Ong, Mol Cell Proteomics (2002), 376-386).

However, the goal must ultimately be to define the cellular concentrations of proteins absolutely, whether as molarities or as numbers of molecules per cell. Abso-

lute quantification, which poses one of the greatest challenges in proteomics, draws on well-established precepts in analytical chemistry, and requires either external standards or internal standards (Sechi, Curr Opin Chem Biol 7 (2003), 70-77; Julka, J Proteome Res 3 (2004), 350-363). External standardization is typified by immunodetection, whether solution phase or on position-addressable antibody arrays (Walter, Trends Mol Med 8 (2002), 250-253; Lopez, J Chromatogr B Analyt Technol Biomed Life Sci 787 (2003), 19-27). The second approach, reliant on internal standardization, is based on mass spectrometry (MS), wherein highly selective detection of ions (or ion fragmentations) characteristic of the analytes of interest is combined with the use of internal standards.

In the most rigorous MS analyses, stable isotopic variants of the analytes are used as internal standards. The key underlying principle is that the determination of *relative* signal intensities during mass spectrometric analysis can be converted into *absolute* quantities of analyte by reference to an authentic standard available in known amounts. Direct application of this approach to intact proteins is impractical, and it is common to adopt the principle of surrogacy, that is to quantify indirectly by reference to a proteolytic peptide derived from the protein of interest.

Analyses based on these principles have been dubbed "AQUA" (absolute quantification) using internal standards synthesized *de novo* by chemical methods (Gerber, PNAS 100 (2003), 6940-6945). However, this approach does not lend itself well to absolute quantification of large numbers of proteins, as each Q-peptide would need to be chemically synthesised and independently quantified.

The international patent application PCT/US03/17686, published as WO 03/102220 provides methods to determine the absolute quantity of proteins present in a biological sample. The principle of WO 03/102220 is based on the generation of an ordered array of differentially isotopically tagged pairs of peptides, wherein each pair represents a unique protein, a specific protein isoform or a specifically modified form of a protein. One element of the peptide pairs is a synthetically generated, external standard and the other element of the pair is a peptide

generated by enzymatic digestion of the proteins in a sample mixture. For performing the method of WO 03/102220 the standard peptides are calibrated so that absolute amounts are known and added for comparison and quantification. A sample of interest is also labelled with the same isotope tag as used for the standard peptides except differing in the isotopic label. The pairs of signals, which correspond to differentially labelled sample and standard peptides are finally observed and related to a list of expected masses based on the particular standard peptides included. The disadvantage of WO 03/102220 is that standard peptides need to be individually synthesised, purified and quantified. Moreover, both the sample and the standard peptides need to be specifically labelled separately, increasing the potential for variability between experiments.

Thus there is still an existing need to develop easy and convenient methods for absolute quantification in proteomics.

Summary of the invention

The present invention is directed to an artificial protein for quantitative analysis of the proteome of a sample, cell or organism, comprising:

(a) at least two consecutive peptides linked by a cleavage sequence for separating the peptides;

(b) a singular marker on one or more peptides for determination of the absolute amount of the protein; and

(c) N-terminal and C-terminal extensions for protection of the peptides;

wherein each peptide represents one single protein of the sample, cell or organism and each peptide is in a defined stoichiometry.

For the purpose of the invention the artificial protein is also named QCAT protein and the peptides used for the QCAT protein are called Q-peptides.

The cleavage sequence between two Q-peptides may be an enzymatic or a chemical cleavage sequence.

The N-terminal and C-terminal extensions protect the quantification peptides from processing and exoproteolysis. The artificial protein further includes features which allow for easy purification of the Q-CAT protein.

Each of the Q-peptides represents one single protein of the sample, cell or organism and each peptide is in a defined stoichiometry, which is typically, but not exclusively 1:1.

The present invention further concerns a collection of Q-peptides, which covers the complete proteome of an organism. This collection allows for rapid quantification of the proteome of such an organism.

The present invention also concerns a vector comprising the QCAT protein and a kit comprising the vector and/or the QCAT protein.

Moreover, the invention is directed to a method for quantitative analysis of the proteome of a sample, cell or organism, comprising the steps of:

(a) quantifying the amount of the protein or one peptide containing the singular marker in an absolute manner;
(b) generating a preparation of the proteins to be quantified;
(c) mixing the products of steps (a) and (b);
(d) completely cleaving the artificial protein of any of claims 1 – 10 and the proteins to be quantified in step (b) at the cleavage sequence;
(e) determining the quantitative amount of peptides;
(f) calculating the absolute amount of peptides,

wherein the artificial protein and/or the peptides are isotopically labelled.

It is important to note that the proteins do not have to be purified – partially purified followed by high resolution separation technologies would be equally acceptable.

Brief description of the drawings

Figure 1 shows examples of Q-peptides selected as signature peptides;

Figure 2 shows the DNA sequence, translated protein sequence and features of the QCAT;

Figure 3 shows the Characterisation of the QCAT protein and Q-peptides;

Figure 4 shows the Quantification using the QCAT protein, and

Figure 5 shows the Use of the QCAT for muscle protein quantification.

Detailed description of the invention

The inventors describe here the design, expression and use of artificial proteins that are concatamers of tryptic Q-peptides for a series of proteins, generated by gene design *de novo*. The artificial protein, a concatamer of Q-peptides ("QCAT") is designed to include both N-terminal and C-terminal extensions. The function of the extensions is to protect the true Q-peptides, to introduce a purification tag (such as a His-tag) and a sole cysteine residue for quantification of the QCAT.

The novel gene is inserted into a high-level expression vector and expressed in a heterologous expression system such as *E. coli*. Within the QCAT protein, each Q-peptide is in a defined stoichiometry (typically, but not exclusively 1:1), such that the entire set of concatenated Q-peptides can be quantified in molar terms by determination of the QCAT protein. Moreover, the QCAT protein is readily produced in unlabelled or labelled form by growth of the expression strain in defined medium containing the chosen label.

The inventors have successfully designed and constructed an artificial gene encoding a concatenation of tryptic peptides (a QCAT protein) from over 20 proteins. The protein further includes features for quantification and purification. The artificial protein was expressed in *E.coli* and synthesis of the correct product was proven by mass spectrometry. The QCAT protein is readily digested with trypsin; is easily quantified and can be used for absolute quantification of proteins. This strategy brings within reach the accurate and absolute quantification of large

numbers of proteins in proteomics studies. Additionally, the QCAT was labelled by selective incorporation of a stable isotope labelled amino acid or by incorporation of ^{15}N nitrogen atoms at every position in the protein.

It is therefore an object of the present invention to provide an artificial protein for quantitative analysis of the proteome of a sample, cell or organism, comprising:

(a) at least two consecutive peptides linked by a cleavage sequence for separating the peptides;

(b) a singular marker on one or more peptides for determination of the absolute amount of the protein; and

(c) N-terminal and C-terminal extensions for protection of the peptides;

wherein each peptide represents one single protein of the sample, cell or organism and each peptide is in a defined stoichiometry.

In a preferred embodiment of the invention, the artificial protein comprises 10 – 200 peptides, preferably 10 – 100 peptides, more preferred 20 – 60 peptides, most preferred 60 peptides. It is possible to include multiple instances of specific peptides to modify the stoichiometry.

In yet another embodiment, the cleavage sequence is cleaved by a protease, preferably by trypsin and the singular marker is a cysteine residue.

Further, one or more peptides of the artificial protein are repeated identically at least one time, preferably one or more times, to achieve a particular stoichiometry between all peptides sequences.

It is preferred to include an affinity tag (e.g. a histidine tag) for purification of the protein. It is particularly preferred to include the affinity tag in either the N-terminal or C-terminal extensions of the protein.

In a further embodiment, the protein is labelled by an isotope, which is selected from the group consisting of ^{13}C, ^{15}N, ^{2}H and ^{18}O.

In yet a further embodiment each peptide comprises between about 3 and 40 amino acids, preferably about 15 amino acids.

The protein may comprise a molecular weight of about 10 – 300 kDa, preferably about 150 – 200 kDa, most preferred about 150 kDa and is preferably expressed in *E. coli.*

The origin of the proteome, i.e. the sample, cell or organism is preferably a mouse, rat, ape or human, but can be from any proteinaceous source.

In a preferred embodiment of the invention, the peptides represent different conformational, metabolic or modification states of the protein, in order to quantify all proteins derived posttranslationally from such a protein.

Further, the peptides of the artificial protein have preferably a defined molecular weight distribution and quantitative ratios. A mass spectrometer can be calibrated, preferably with molecular weights, which result in equidistant mass spectroscopy signals. Preferably, one or more peptides are represented twice in order to unambiguously label a reference molecular weight for calibration.

The invention is further directed to a collection of peptides, as defined in step (b) of claim 1, which covers the complete proteome of an organism. All expressed proteins of an organism are defined as the proteome of such organism. This allows for rapid quantification of the proteome of such organism.

Further included in the disclosure of the present invention is the absolute quantification of the protein levels of a certain reference strain, which can then be used to compare the protein levels of this particular strain or similar strains under varying experimental conditions.

The invention is also directed to a vector comprising a nucleic acid encoding the artificial protein and a kit comprising the artificial protein and/or the nucleic acid encoding the artificial protein. Further the invention is directed to a method for quantitative analysis of the proteome of an organism, which is described in detail above.

The present invention will be better understood by the encompassed examples and results with reference to the accompanying figures.

Detailed description of the figures

Figure 1. Examples of Q-peptides selected as signature peptides.

For a series of proteins, peptides were selected (using multiple criteria) from proteins identified as the abundant proteins in a soluble fraction of chicken skeletal muscle, and assembled into an artificial protein, or Q-cat. Left side: Coomassie blue stained, SDS-PAGE analysis of soluble chicken muscle proteins. Center: MALDI-ToF spectrum of tryptic digestion of gel slices corresponding to selected protein bands. The peptide ion labelled with an oval represents the peptide chosen for inclusion in the Q-cat protein, Right side: mass and position of the indicated signature peptides within the designed Q-cat protein. Details of the peptides are in Table 1.

Figure 2. The DNA sequence, translated protein sequence and features of the QCAT.

The DNA sequence of the synthetic gene, with relevant cloning sites, is shown on the top line and the derived amino acid sequence is shown below. The grey blocked areas indicate the extent of the tryptic peptides, with the donor chicken proteins, tryptic peptide assignment (T1-T25) and the peptide mass (in Da) indicated. A non-cleavable Arg-Pro tryptic site within phosphoglycerate kinase (boxed) is included to confirm the non-digestibility of this site. Peptides (white boxes) encode the initiator methione, N-terminal sacrificial sequence and spacer se-

quences, and are not derived from proteins of interest. The black boxes highlight the sequences carrying the unique cysteine residue for quantification and His_6 tag for purification. T1 and T2 are sacrificial peptides designed to protect the N-terminus of the first true Q-peptide (T3)

Figure 3. Characterisation of the QCAT protein and Q-peptides

The pET21a/QCAT plasmid was transformed into *E. coli* DE3 cells and after a period of exponential growth, the expression of the QCAT was induced with IPTG. The cell lysates from pre-induced and induced cells were compared on SDS-PAGE (inset). After solubilization of the pellet, and affinity chromatography on a NiNTA column, the purified QCAT protein was homogeneous, and was digested in solution with trypsin. The peptides were analysed on MALDI-ToF mass spectrometry. The inset tryptic digestion map is shaded to indicate the relative intensities of signals corresponding to each peptide in the mass spectrum; peptides smaller than 900 Da, derived from the 'sacrificial' parts of the QCAT are less readily detected in this type of mass spectrometric analysis due to interfering ions.

Figure 4. Quantification using the QCAT protein

The QCAT protein was prepared in unlabelled form (L: "light") and in a form uniformly labelled with ^{15}N (H: "heavy"). The H and L QCAT proteins were separately purified, quantified and mixed in different ratios, before tryptic digestion and measurement of peptide intensities by MALDI-ToF mass spectrometry. Panel **a**) illustrates the mass spectrum for the Q-peptide for adenylate kinase (GFLIDGYPR, 12 nitrogen atoms). In panel **b**) the measured L:H ratios were plotted relative to the mixture ratio, in a triplicate series of experiments for which individual points are shown. In the bottom panel, the data for seven peptides are collated and expressed as mean ± SD (n=18-21). The dotted line defines the 95% confidence limits of the fitted straight line.

Figure 5. Use of the QCAT for muscle protein quantification.

A preparation of soluble proteins from skeletal muscle of chicks at 1d and 27d was mixed with [^{15}N] QCAT, digested with trypsin and analysed by MALDI-ToF MS. For a subset of proteins, it was possible to determine the intensities of the endogenous and standard peptide, and from this, calculate the absolute amounts (in nmol/g tissue) of each protein. Three animals were used at each time point, error bars are SEM (n=3). Proteins were AK: adenylate kinase, ApoA1: apoliporotein A1, LDHB: lactate dehydrogenase B, Beta Trop: beta tropomyosin, Beta Eno: beta enolase, GP: glycogen phosphorylase, ALDO B: aldolase B, TPI: triose phosphate isomerase, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, Actin, API: actin polymerization inhibitor, PK: pyruvate kinase and CK: creatine kinase.

1. Design of the gene encoding the Q-protein concatamer

One of the inventors' major interests is in proteome dynamics (Pratt, Mol Cell Proteomics 1 (2002), 579-591), and in changes in protein expression during muscle development (Doherty, Proteomics 4 (2004), 2082-2093; Doherty, Proteomics in press (2005)). A system that shows dramatic developmental changes in protein expression is the chicken pectoralis skeletal muscle from immediately post-hatching to maturity. Accordingly, for the demonstration QCAT set, the inventors chose twenty chicken proteins that had been previously identified as changing in expression level in developing skeletal muscle (Doherty, Proteomics 4 (2004), 2082-2093). A single tryptic fragment was chosen to represent each protein (a "Q-peptide"), although a peptide that can be reproducibly generated by any proteolytic or chemical fragmentation could be used, and in this example, Q-peptide selection was based on theoretical and experimental criteria. The first criterion was that the Q-peptides should lack a cysteine residue, as cysteine residue could be used for quantification of the QCAT and the absence of cysteines should avoid complex intra- and inter-molecular disulphide bond formation in the expressed protein. Secondly, the peptide chosen should be unique within the set of Q-peptides. Thirdly, the Q-peptides were chosen with masses between 1000 Da and 2000 Da, corresponding to the region in MALDI-ToF mass spectra where sensitiv-

ity of detection is typically high and interfering signals are low. Finally, an operational criterion was added, inasmuch as the inventors selected peptides that were already demonstrated to give a strong signal on MALDI-ToF mass spectrometry 75% (15 out of 20) of which were Arg-terminated tryptic peptides - the propensity of such peptides to give stronger signals on MALDI-ToF mass spectrometry is well documented (Brancia, Electrophoresis 22 (2001), 552-559). A final, less important criterion was that the Q-peptides should contain at least one instance of an abundant and chemically refractory amino acid such as leucine or valine, as this would facilitate metabolic labeling with amino acids for the preparation of stable isotope labeled Q-peptides. The peptides are summarized in Table 1:

Table 1 - Peptides selected for Q-cat protein

	Peptide mass (Da)	Sequence	N atoms	Parent protein
T1	405.2	MAGK	5	Construct, sacrificial
T2	386.25	VIR	6	Construct, sacrificial
T3	1036.52	GFLIDGYPR	12	Adenylate kinase
T4	1601.87	VVLAYEPVWAIGTGK	16	Triose phosphate isomerase
T5	1176.57	NLAPYSDELR	14	Apolipoprotein A1
T6	1193.56	GDQLFTATEGR	15	Myosin binding protein C
T7	1789.88	SYELPDGQVITIGNER	21	Alpha actin
T8	1291.67	QVVESAYEVIR	15	Lactate dehydrogenase B
T9	1390.74	LITGEQLGEIYR	16	Beta enolase
T10	1361.63	ATDAESEVASLNR	17	Alpha tropomyosin
T11	1160.58	SLEDQLSEIK	12	Myosin heavy chain (embryonic)
T12	1441.68	VLYPNDNFFEGK	15	Glycogen phosphorylase
T13	1489.71	GILAADESVGTMGNR	19	Aldolase B
T14	1345.64	ATDAEAEVASLNR	17	Beta tropomyosin
T15	1687.8	LQNEVEDLMVDVER	19	Myosin heavy chain (adult)
T16	1748.77	LVSWYDNEFGYSNR	19	Glyceraldehyde 3-phosphate dehydrogenase
T17	1767.98	ALESPERPFLAILGGAK	21	Phosphoglycerate kinase
T18	1249.65	QVVDSAYEVIK	13	Lactate dehydrogenase A
T19	1803.93	AAVPSGASTGIYEALELR	21	Alpha enolase

T20	1823.97	LLPSESALLPAPGSPYGR	21	Actin polymerization inhibitor
T21	1857.9	FGVEQNVDMVFASFIR	25	Pyruvate kinase
T22	1991.95	GTGGVDTAAVGAVFDISNADR	25	Creatine kinase
T23	274.15	AGK	4	Construct, sacrificial
T24	892.42	VICSAEGSK	10	Construct, quantification
T25	1408.68	LAAALEHHHHHH	24	Construct, purification tag

Once the candidate set was nominated, the Q-peptides were assembled and a gene was constructed, which encoded the assembled Q-peptides using codons for maximal expression in *E. coli.* At the C-terminus an extension was added to provide a cysteine residue and a His tag purification motif (the latter provided in this case by the vector pET21a). An additional series of amino acids was appended to the N-terminus to provide an initiator methionine residue and a sacrificial peptide, which when cleaved would expose a true Q-peptide (Figure 1). This avoided complications due to N-formylation or removal of methionine from the N-terminus of QCAT. The transcript encoded by the initial QCAT gene was then analyzed *in silico* for features such as hairpin loops that might compromise translation. If such a feature was noted, the order of the Q-peptides was swapped until an acceptable mRNA structure was obtained – the sequence of Q-peptides within a QCAT is not relevant to their use as quantification standards and the order is thus amenable to such manipulation. The gene was constructed from a series of overlapping oligonucleotides and confirmed by DNA sequencing.

2. Expression of the QCAT

The QCAT gene was constructed with restriction sites, such that it could be inserted into a range of expression vectors (Figure 2). In this instance, the gene (confirmed by DNA sequencing) was inserted into pET21a at the *Nde*I and *Hind*III sites and was expressed initially in *E.coli* (NovaBlue (DE3)) grown in rich medium. After induction by IPTG, SDS-PAGE analysis confirmed high-level expression of a protein of the expected mass (~35 kDa). This protein was present in the insoluble fraction of sonicated cells, and was presumed to be the QCAT protein present in inclusion bodies. From this preparation we purified the QCAT protein by affinity

chromatography using Ni-NTA resin, which resulted in a homogeneous preparation (Figure 2, inset). The intact average mass of this protein, measured by ESI-MS was 33036±2 Da (data not shown), compared to the predicted mass of 33167 Da for the QCAT protein, a difference of 131 Da which is exactly consistent with loss of the methionine residue from the N-terminus. The approx. 35 kDa gel band was subjected to in-gel digestion with trypsin and analysed by MALDI-ToF MS. All predicted QCAT peptides were readily observed in the MALDI-ToF mass spectrum, although the N and C-terminal sacrificial peptidic material yielded, by design, fragments that were too small to be seen in the MALDI-ToF mass spectrum (Figure 3). Although the peptides were chosen to yield good signals on MALDI-ToF, some peptides were markedly less intense than others. These included all of the lysine-terminated peptides, with the exception of T17, which included a non-cleavable Arg-Pro site, which although lysine terminated still yielded a strong signal on MALDI-ToF mass spectrometry. This was particularly evident in the isoform-specific Q-peptides T8 and T18, derived from lactate dehydrogenases A and B (QVVESAYEVIR and QVVDSAYEVIK respectively) - the lysine terminated peptide was less than 10 % of the intensity of the arginine-terminated peptide, which suggests that either Q-peptides should be predominantly drawn from arginine terminated peptides, or alternatively, that a step such as guanidination should be used to convert lysine residues to homoarginine residues, enhancing the propensity to give strong signals (Brancia, Electrophoresis 22 (2001), 552-559). The QCAT was digested by trypsin very effectively and there was no evidence for partial proteolytic products of the Q-peptides, which would of course compromise the quantification step. The inventors then expressed the protein in minimal medium containing $^{15}NH_4Cl$ as sole nitrogen source. When digested with trypsin, the resultant MALDI-ToF mass spectrum was of high quality, and all Q-peptides were detectable at the appropriate mass shift corresponding to the number of nitrogen atoms in the peptide (data not shown).

The unlabelled and ^{15}N-labelled QCAT proteins were then mixed in different ratios, and digested with trypsin before the resultant limit peptides were analysed by MALDI-ToF mass spectrometry. The heavy and light variants of the peptides were

readily discerned (Figure 3a) and their intensities measured for a series of peptides (Figure 3b). In all instances, the data were of high quality, and the relationship between proportion of material and the heavy:light ratios was linear, with a slope of one (mean ± SD (n=6) = 1.008 ± 0.008), and very high correlation coefficients (r^2 greater than 0.99 in all instances). The combined data (Figure 3c) expresses the data for seven peptides; the close boundaries defined by the 95% confidence limits indicate the quality of the quantification.

Summary of results

The inventors have applied this particular QCAT in the analysis of protein expression in chick skeletal muscle, at 1 d and 27 d post-hatching (Figure 4). Twelve proteins present in this preparation were also represented in the QCAT. MALDI-ToF data of the tryptic peptides was readily acquired, and the changes in protein levels that occur over the first three to four weeks post-hatching were determined.

Because the proteins were absolutely quantified, the inventors were able to express the proteins as nmol per g wet weight of tissue. The variance of the triplicate analyses was small; the inventors attribute this variance to biological rather than analytical variation. The inventors have previously measured the levels of seven of these proteins by 2D gel electrophoresis and densitometry, and the correlation between the quantification using both methods was 0.82 (r^2, $p>0.001$). Recognizing that the two methods assess different representations of the proteome, such as charge-variant isoforms or total protein complement, and that the densitometirc method is inevitably imprecise, the correlation is good.

The inventors have demonstrated the feasibility of the QCAT approach for generation of a concatenated set of Q-peptides. The QCAT, designed using both theoretical and experimental considerations, was expressed at high levels, even when grown on minimal medium, and the product was successfully purified. Because the QCAT is a completely artificial construct, the inventors did not anticipate that it would fold into any recognizable three-dimensional structure, and as expected, the protein aggregated into inclusion bodies. This is an advantage as subsequent pu-

rification is simpler, only requiring resolubilization of the pellet in strong chaotropes prior to affinity purification. Further, the lack of higher order structure of the QCAT would ensure that the QCAT was digested at least as quickly as the target proteins to be quantified.

Use of the artificial protein and the QCAT peptides

There are two ways in which the concatamers will be used. First, in the **direct Q-peptide** method, the concatamers are used as an internal standard. The stable-isotope labelled concatamer can be directly added to a sample or cell preparation before the proteolysis step. Alternatively, for some cell systems, the concatamer quantification can be used to achieve absolute quantification of a reference strain grown under carefully defined conditions - the **indirect Q-peptide** approach. This reference strain can then be used, in stable isotope labelled form, as an absolute quantification standard for all future proteomics quantification studies using that organism. Once a reference strain is accurately quantified, any peptide can be used to report on a protein, rather than the restricted set used as Q-peptides and this is clearly a very attractive proposition. This extends the generality of different proteomic strategies, and creates a new niche for tagging methods such as ICAT (Gygi, J Proteome Res 1 (2002), 47-54) and ITRAQ (Ross, Molecular and Cellular Proteomics in press (2004)) in a comparative proteomics analysis of an unknown against a fully quantified strain.

However, the inventors recognize that there are many instances where this approach is not appropriate, and where a stable isotope labelled concatamer itself (the **direct Q-peptide** approach) will be the appropriate standard. This is particularly apposite in proteomics studies using biological material that cannot be readily pre-labelled, for example, in animal tissues or in biomarker studies.

Particular advantages

The strategy the inventors advocate is superior to chemical synthesis of each individual Q-peptide, usually in a stable isotope form. Whilst chemical synthesis has been used in one-off applications, the process of peptide synthesis is not suffi-

ciently 'clean' as to obviate exhaustive purification of the product. Secondly, for multiplexed assays, each peptide would need to be individually quantified before use. Finally, chemically synthesized Q-peptides are a finite resource whereas repeated expression of the QCAT gene is facile. High quality absolute quantification may be an effective route to overcome the difficulties associated with current methods for comparative proteomics, whether based on gel analysis or mass spectrometry. A series of comparative studies of particular cellular systems, each by comparison to a QCAT quantified reference would not only be individually quantified, but should be sufficiently rigorous that as the data sets grow, any pairwise comparison would be robust, transferable between individual laboratories and stable over time.

It should be possible to factor in the propensity of the peptide to ionize and generate a good signal in the mass spectrometer. At present, ion intensities are not used exhaustively in the analysis of mass spectra, although there have been some recent attempts to predict intensity using knowledge-based approaches (Krause, Anal Chem 71 (1999), 4160-4165; Gay, Proteomics 2 (2002), 1374-1391; Baumgart, Rapid Commun Mass Spectrom 18 (2004), 863-868).

An additional factor that must be taken into account is the choice of precursor label. Whilst uniform labeling with [^{13}C] or [^{15}N] ensures that every peptide is comprehensively labeled, it might be preferable to select Q-peptides that each contain the same amino acid that is then used as the stable isotope labeled precursor. Since most QCAT proteins would be anticipated to be assemblies of tryptic peptides, a strategy of incorporation of [$^{13}C_6$]-lysine and [$^{13}C_6$]-arginine would also ensure that most Q-peptides would be singly labeled and the mass offset between heavy and light peptides would be a constant 6 Da. Further, an unlabelled QCAT could be labeled *in vitro* using reagents advocated for comparative proteomics, enhancing all of these technologies to absolute quantification.

Without being bound by any particular theory, the inventors believe that the number of Q-peptides that could be assembled into a single QCAT is limited by the

ability to achieve high-level heterologous expression of large proteins. In the example given here, the inventors chose 20 peptides of average length 15 amino acids and average molecular weight 1.5 kDa. If 100 proteins were represented in a single QCAT, the resultant recombinant protein would be 150 kDa, which should be readily expressed. The entire yeast proteome, of approx 6000 proteins, could then be defined within approx. 60 QCAT constructs.

This ability to quantify as many as 100 proteins in a single construct invites the challenge of optimal assembly of individual Q-peptides in QCATs. Different criteria might be envisaged. First, a group of Q-peptides would allow absolute quantification of a particular subcellular fraction, or of a specific subset of proteins, for example transcription factors or protein kinases. Secondly, and perhaps more importantly, concatenation could be driven by the abundance of the target proteins in the cell. It would be difficult to quantify two different proteins with widely different expression levels in the same QCAT experiment, and it might be preferable to assemble high abundance proteins in a construct distinct from that encoding low abundance proteins.

Other applications for QCATs are readily envisaged, particularly in the broad areas of clinical biology and toxicology and diagnostics. Absolute quantification will add a new dimension to the predictive values of analyses in clinical or other biomarker monitoring systems and will absolutely define the stoichiometric ratios of individual proteins within a subcellular compartment or a multi-protein complex.

Materials and methods

Materials. $[^{15}N]H_4Cl$ (99% atom percent excess) was provided by CK Gas Products Ltd. Hampshire, UK. Most reagents, except where listed here, have been described previously (Doherty, Proteomics 4 (2004) 2082-2093; Pratt, Proteomics 2 (2002), 157-160). Chick (layer, Hi-Sex Brown) skeletal muscle proteins were prepared from 1 d and 27 d old chicks as a 20.000g supernatant of a 10 % (w/v) ho-

mogenate (Doherty, Proteomics 4 (2004) 2082-2093; Doherty, Proteomics 5 (2005) 5, 522-533.

QCAT gene design and construction. Q-peptides were selected for uniqueness of mass, propensity to ionise and be detectable in mass spectrometry, the presence of specific amino acid residues (for example, leucine or valine), the absence of other amino acid residues (cysteine, histidine, methionine). The peptide sequences were then randomly concatenated *in silico* and used to direct the design of a gene, codon-optimised for expression in *E. coli*. The predicted transcript was analysed for RNA secondary structure that might diminish expression, and if this was present, the order of the peptides was altered. N-and C-terminal sequences were added as sacrificial structures, protecting the assembly of true Q-peptides from exoproteolytic attack during expression. Additional peptide sequences were added to provide an initiator methionine and a C-terminal cysteine residue for quantification. The artificial gene was synthesised *de novo* (by Entelechon GmbH, Germany) from a series of overlapping oligonucleotides, verified by DNA sequencing and ligated into the *Nde*I and *Hind* III sites of the pET21a expression vector, to yield the QCAT plasmid, pET21a QCAT. A His_6 purification tag was provided by fusion to the vector.

QCAT gene expression and labeling with ^{15}N. The QCAT plasmid, pET21a QCAT, was used to transform NovaBlue (DE3) (K-12 *endA1, hsdR17*($r_{K12}^{-}m_{K12}^{+}$), *supE44, thi-1, recA1, gyrA96, relA1, lac*, F'[*proA*$^{+}$*B*$^{+}$, *lacI*^{q}*Z*□*M15* ::Tn*10*(Tc^{R})] cells to ampicillin resistance. Cells were grown at 37 °C in Luria broth, 100 µg/ml ampicillin to an A_{600} of 0.4-0.6 and IPTG added to 1 mM. Incubation continued for a further five hours when cells were pelleted by centrifugation (5.000g, 4 min., 4°C), resuspended in 10 ml 20 mMTris/HCl buffer, pH 8.0 and lysozyme was added (100 µg/ml) for ten minutes at room temperature. Cells were then sonicated (three bursts of 30s) on ice and centrifuged at 14000 **g** for 10min. Pellets and supernatants of induced and uninduced cultures were analysed by 12,5 % (w/v) SDS PAGE/Coomassie blue staining. For ^{15}N-labelling, cells were grown in M9 minimal medium prepared using [^{15}N]H_4Cl (20 mM), and induced and processed as above.

Purification and analysis of the QCAT protein. The pellets from sonicated cells were dissolved in 20 mM phosphate buffer (pH 7.5) containing 20 mM imidazole and 8 M urea (Buffer A) before being applied to a NiNTA column (GE Healthcare). After 10 column volumes of washing in the same buffer, the bound material was eluted with buffer A with an increased concentration of imidazole (500 mM). This material was desalted on Sephadex G25 'spun columns' and the mass of the eluted protein was determined by electrospray ionisation mass spectrometry using a Waters-Micromass Q-ToF micro mass spectrometer. The mass spectra were processed using the MaxEnt I algorithm. The purified desalted protein was digested with trypsin, and the resultant peptides were mass measured using a Waters-Micromass MALDI-ToF mass spectrometer (Doherty, Proteomics 4 (2004) 2082-2093). To assess the response ratio of heavy and light variants of the QCAT, the purified 'heavy' and 'light' proteins were mixed in different ratios prior to digestion with trypsin and MALDI-ToF mass spectrometry. The intensities of the [^{14}N]- and [^{15}N]- peptides were measured on centroided spectra.

Use of the QCAT to quantify muscle protein expression. The supernatant fraction containing chicken soluble proteins derived from 100mg of tissue was mixed with 290 µg of [^{15}N] QCAT, quantified by protein assay and digested with trypsin overnight – the QCAT was digested at a higher rate than endogenous muscle proteins (results not shown). The experiment was replicated for three animals at each time point. Subsequently, the [^{14}N]- (muscle) and [^{15}N]- (QCAT) peptides were identified by mass, and their relative intensities measured by MALDI-ToF mass spectrometry.

Mass Spectrometric Analysis

The analysis in this example was carried out using a MALDI-ToF Mass Spectrometer, but this method is equally applicable to all other Mass Spectrometric methods suitable for the analysis of peptides.

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WO 03/102220 (Aebersold, R.)

Claims

1. Artificial protein for quantitative analysis of the proteome of a sample, cell or organism, comprising:
   (a) at least two consecutive peptides linked by a cleavage sequence for separating the peptides;
   (b) a singular marker on one or more peptides for determination of the absolute amount of the protein; and
   (c) N-terminal and C-terminal extensions for protection of the peptides;
   wherein each peptide represents one single protein of the sample, cell or organism and each peptide is in a defined stoichiometry.

2. Artificial protein of claim 1, wherein the protein comprises 10 – 200 peptides, preferably 10 – 100 peptides, more preferred 20 – 60 peptides, most preferred 60 peptides.

3. Artificial protein of claim 1 or 2, wherein the cleavage sequence is cleaved by a protease, preferably by trypsin.

4. Artificial protein of any of the preceding claims, wherein the singular marker is a cysteine residue.

5. Artificial protein of any of the preceding claims, wherein one or more peptides are repeated identically one or more times, in order to achieve a particular stoichiometry between all peptide species.

6. Artificial protein of any of the preceding claims, wherein the protein comprises an affinity tag for purification of the protein.

7. Artificial protein of any of the preceding claims, wherein the protein is labelled by an isotope.

8. Artificial protein of any of the preceding claims, wherein each peptide comprises between about 3 and 40 amino acids, preferably about 15 amino acids.

9. Artificial protein of any of the preceding claims, wherein the peptides represent different conformational, metabolic or modification states of the protein.

10. Artificial protein of any of the preceding claims, wherein the peptides have a defined molecular weight distribution and quantitative ratios.

11. A collection of peptides as defined in step (b) of claim 1, which covers the complete proteome of an organism to allow the rapid quantification of the proteome of such an organism.

12. Vector comprising a nucleic acid encoding the artificial protein of any of claims 1 – 10.

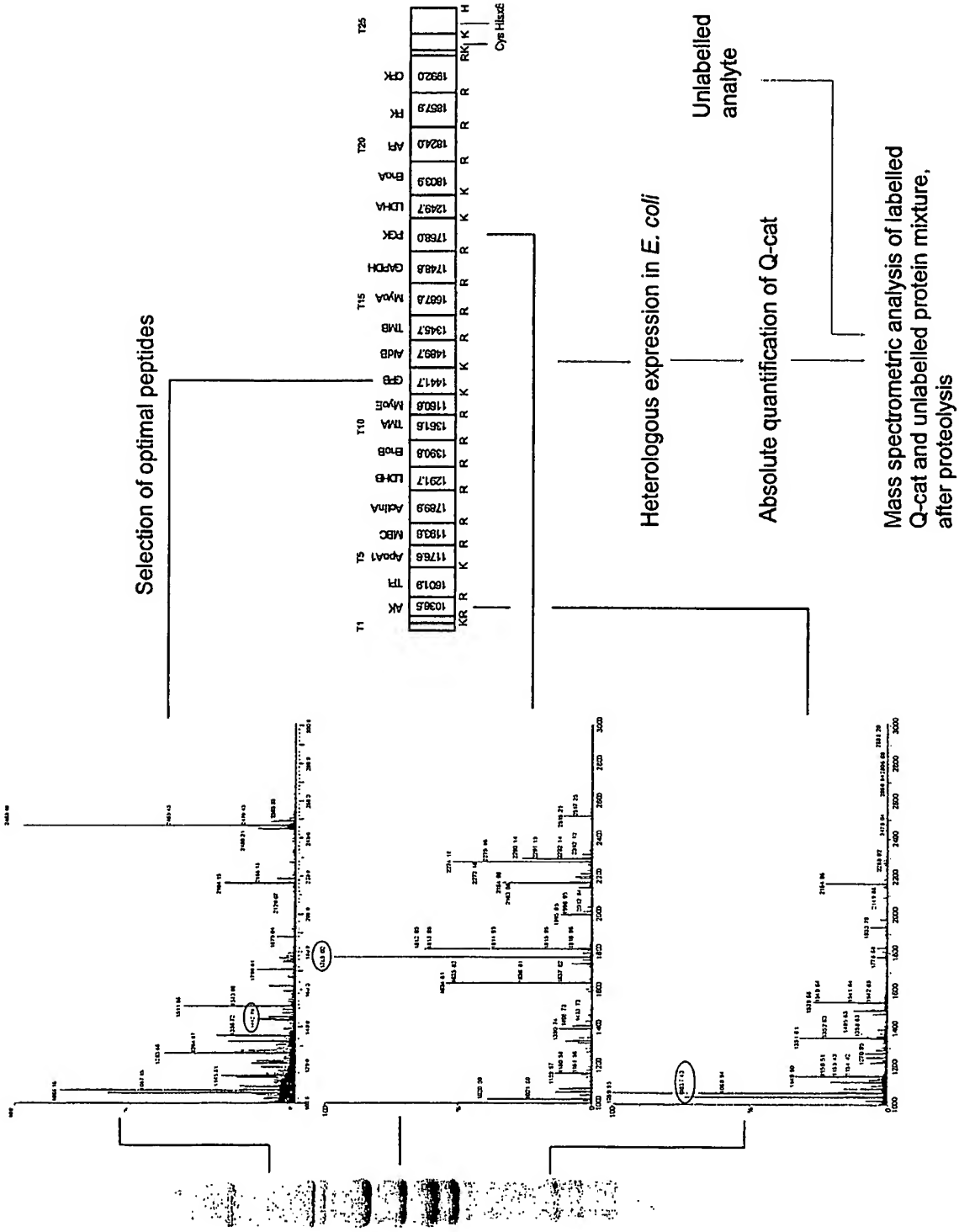
13. Kit comprising the vector of claim 12 and/or the artificial protein of any of claims 1 – 10.

14. A method for quantitative analysis of the proteome of a sample, cell or organism, comprising the steps of:

(a) quantifying the amount of the protein or one peptide containing the singular marker in an absolute manner;
(b) generating a preparation of the proteins to be quantified;
(c) mixing the products of steps (a) and (b);
(d) completely cleaving the artificial protein of any of claims 1 – 10 and the proteins to be quantified in step (b) at the cleavage sequence;
(e) determining the quantitative amount of peptides;
(f) calculating the absolute amount of peptides,

wherein the artificial protein and/or the peptides are isotopically labelled.

FIG. 1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Unlabelled analyte
Absolute quantification of Q-cat
Mass spectrometric analysis of labelled Q-cat and unlabelled protein mixture, after proteolysis
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TPI
ApoA1
MBC
Actin
LDH-B
Enol
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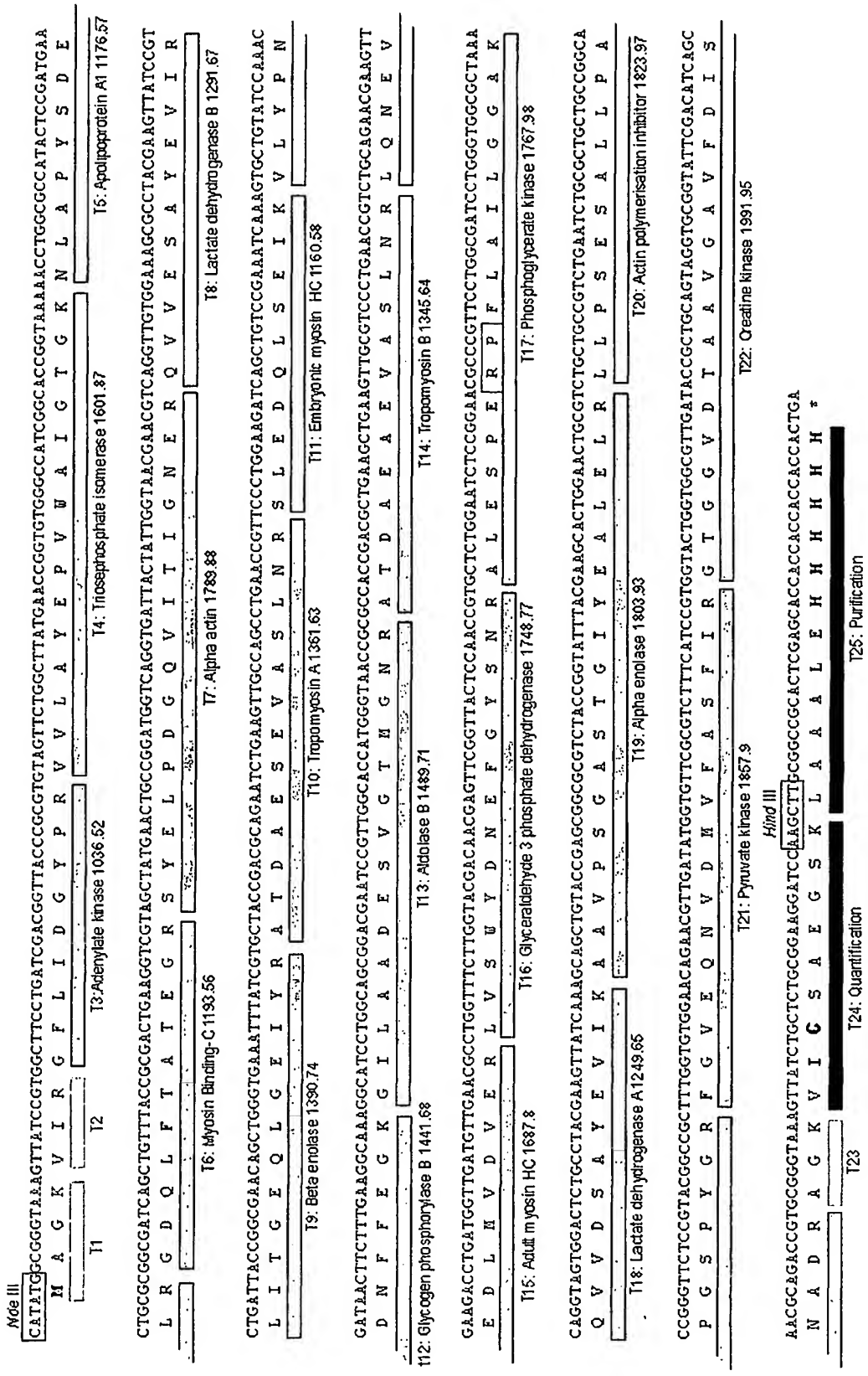
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T1
T2
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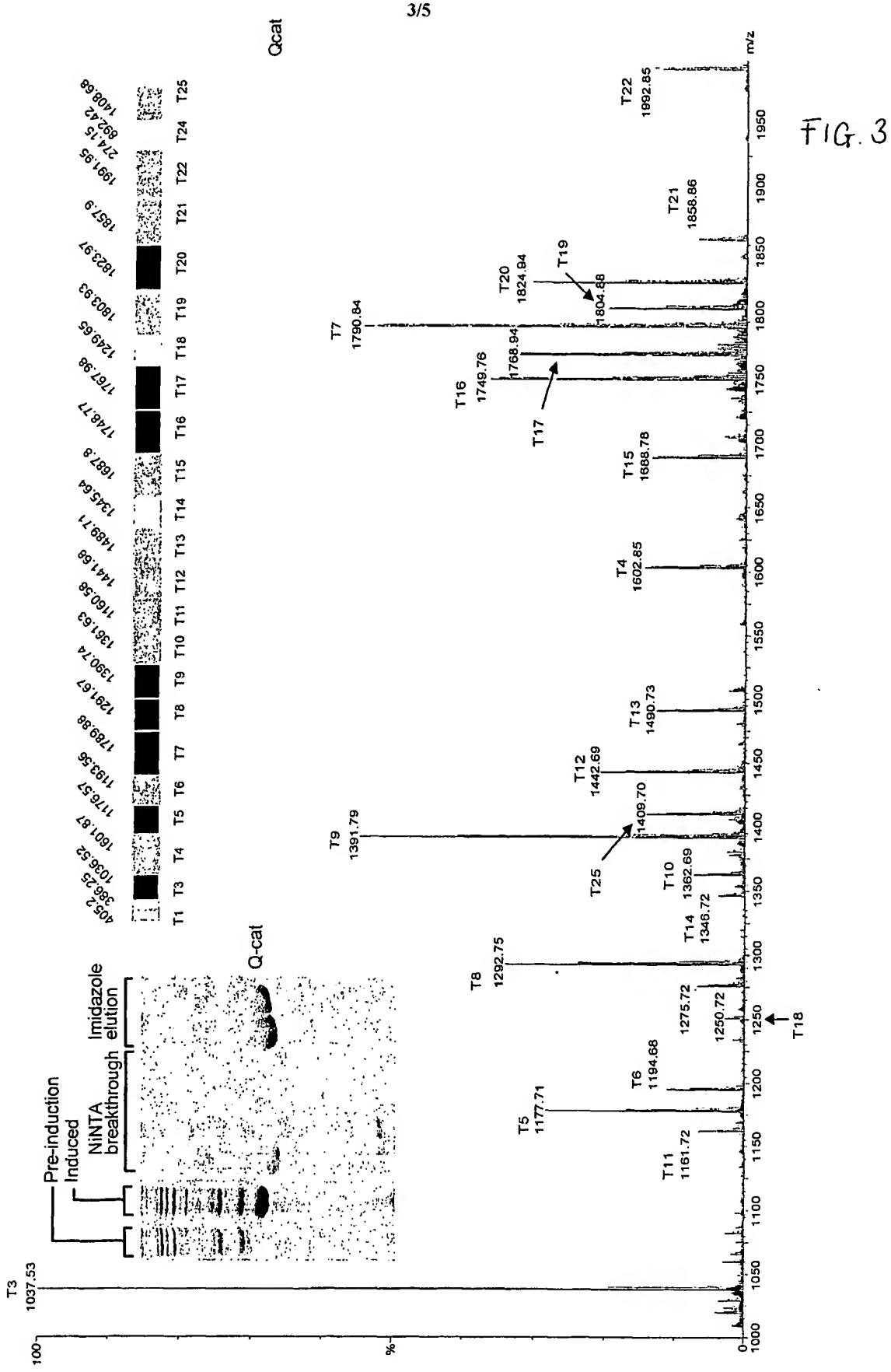
T7: Alpha actin 1789.88
T8: Lactate dehydrogenase B 1291.67
T9: Beta enolase 1390.74
T10: Tropomyosin A 1361.63
T11: Embryonic myosin HC 1160.58
T12: Glycogen phosphorylase B 1441.68
T13: Aldolase B 1489.71
T14: Tropomyosin B 1345.64
T15: Adult myosin HC 1687.8
T16: Glyceraldehyde 3 phosphate dehydrogenase 1748.77
T17: Phosphoglycerate kinase 1767.98
T18: Lactate dehydrogenase A 1249.65
T19: Alpha enolase 1803.93
T20: Actin polymerisation inhibitor 1823.97
T21: Pyruvate kinase 1857.9
T22: Creatine kinase 1991.95
Hind III
T23
T24: Quantification
T25: Purification

FIG. 2


Qcat
Q-cat
Pre-induction
Induced
NiNTA breakthrough
Imidazole elution
m/z
%
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T3 1037.53
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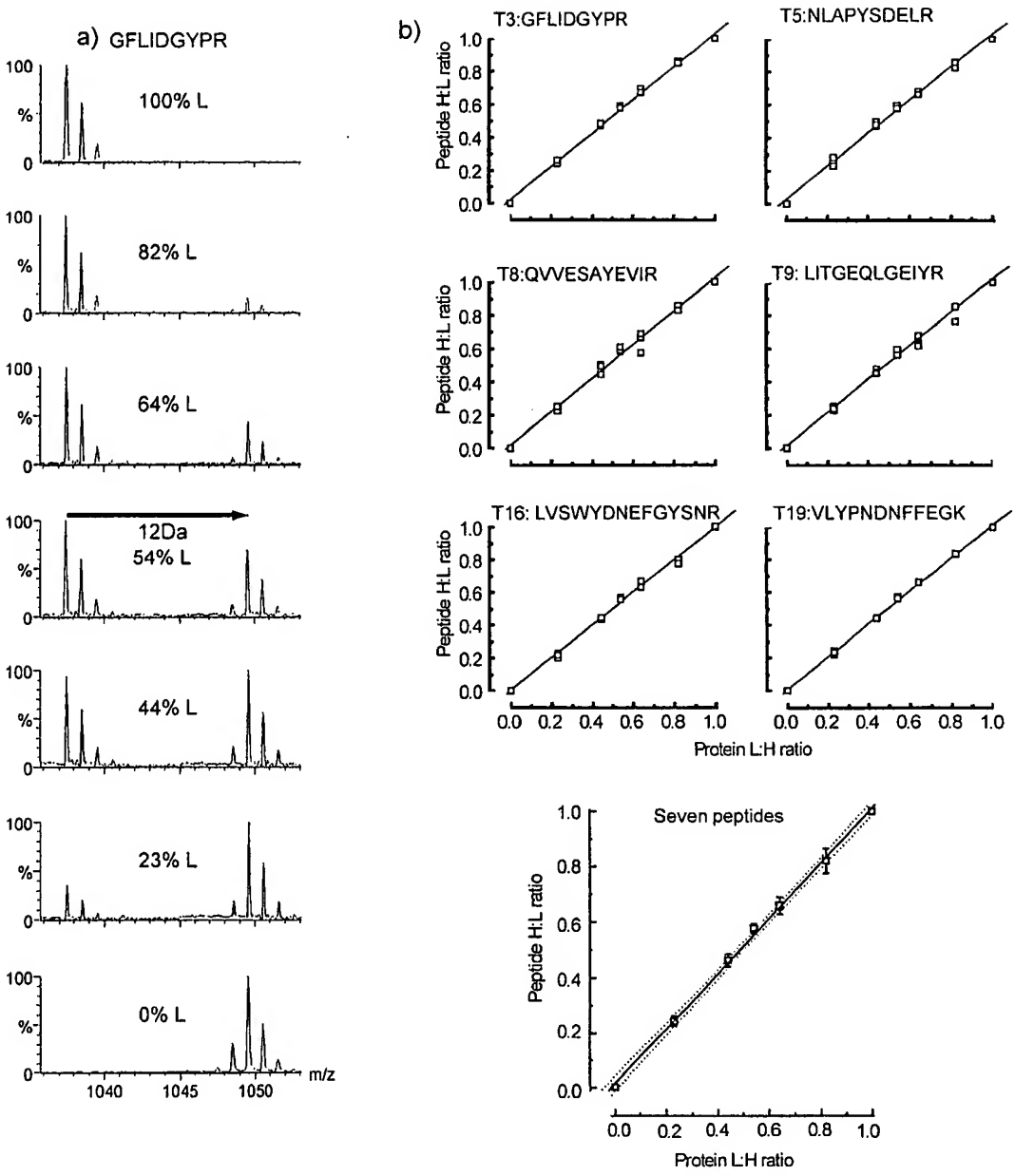
FIG. 3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12Da
54% L
44% L
23% L
0% L
100
%
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1040
1045
1050
m/z
b)
T3:GFLIDGYPR
T5:NLAPYSDELR
T8:QVVESAYEVIR
T9: LITGEQLGEIYR
T16: LVSWYDNEFGYSNR
T19:VLYPNDNFFEGK
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Protein L:H ratio
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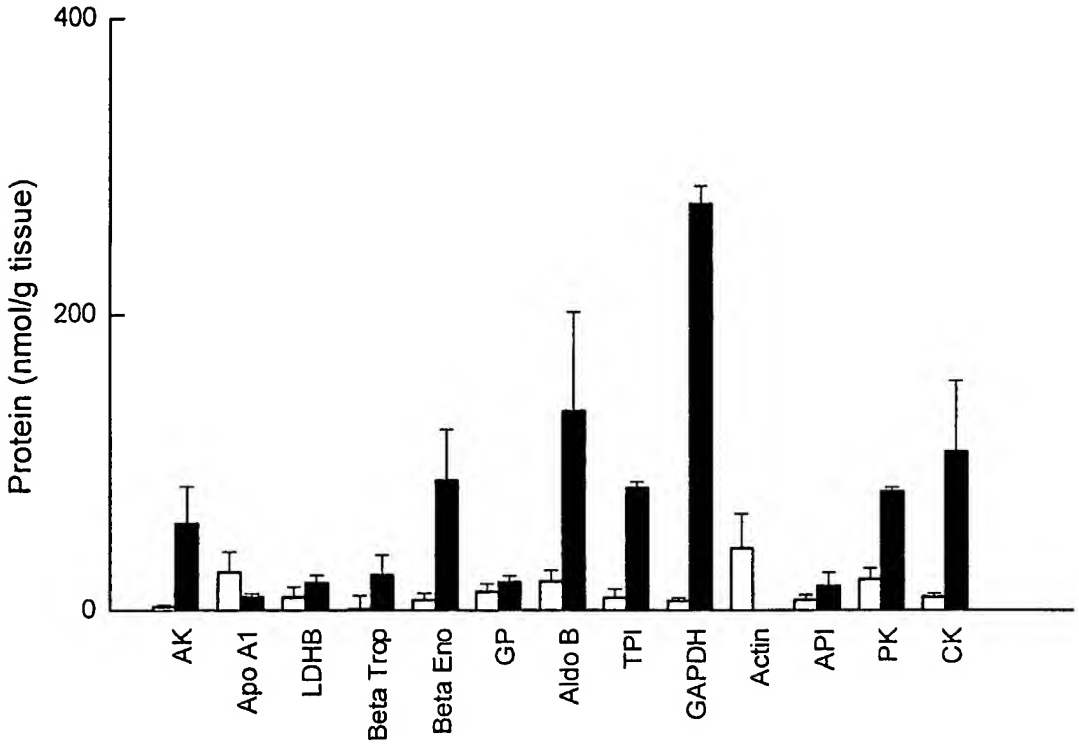
0.4
0.6
0.8
1.0
Seven peptides

FIG. 4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200
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Protein (nmol/g tissue)
AK
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LDHB
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GAPDH
Actin
API
PK
CK

FIG. 5

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2005/012530

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K7/08 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/013636 A (SYNGENTA PARTICIPATIONS AG) 12 February 2004 (2004-02-12) pages 4,5 pages 11-13 page 16, lines 18-25 page 20, lines 14-20 pages 46,47,52 claims 1,18 -----	1-13
X	WO 03/102220 A (THE INSTITUTE FOR SYSTEMS BIOLOGY; AEBERSOLD, RUDOLF, H) 11 December 2003 (2003-12-11) cited in the application pages 8,9 claims 8,10 ----- -/--	1-14

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 April 2006	11/05/2006

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Boiangiu, C

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2005/012530

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2002/037532 A1 (REGNIER FRED E ET AL) 28 March 2002 (2002-03-28) paragraphs [0023] - [0027], [0053], [0058], [0062]; claims 1-4; figure 1	1-14
A	US 2004/229283 A1 (GYGI STEVEN P ET AL) 18 November 2004 (2004-11-18) abstract; claim 1	1-14
T	ROBERT J BEYNON, MARY K DOHERTY, JULIE M PRATT & SIMON J GASKELL: "Multiplexed absolute quantification in proteomics using artificial QCAT proteins of concatenated signature peptides" NATURE METHODS, vol. 2, no. 8, August 2005 (2005-08), pages 587-589, XP008051422 the whole document	1-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2005/012530

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004013636 A	12-02-2004	AU 2003256016 A1	23-02-2004
		EP 1525483 A2	27-04-2005
WO 03102220 A	11-12-2003	AU 2003259029 A1	19-12-2003
US 2002037532 A1	28-03-2002	US 2003129769 A1	10-07-2003
		US 2003092076 A1	15-05-2003
US 2004229283 A1	18-11-2004	NONE	